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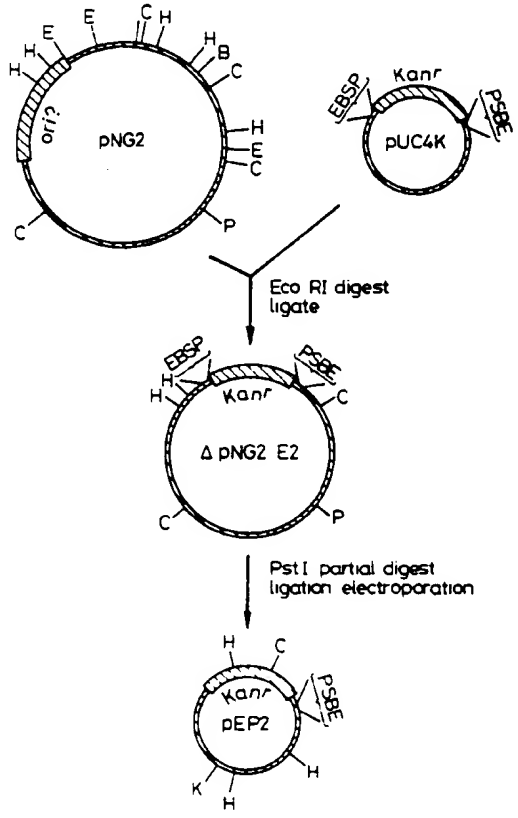
(54) Title: SHUTTLE PLASMID FOR ESCHERICHIA COLI AND MYCOBACTERIA

(57) Abstract

DNA shuttle vectors which are capable of replication in Mycobacteria, *E. coli* and other bacterial hosts are described. The shuttle vectors comprise a single origin of replication which confers the ability of replication in a number of bacterial species. Also disclosed are shuttle vectors encoding desired polypeptides, such as antigens of disease causing bacteria, viruses and parasites.



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# SHUTTLE PLASMID FOR ESCHERICHIA COLI AND MYCOBACTERIA

This invention relates to DNA vectors, and is particularly concerned with DNA shuttle vectors which are capable of replication in mycobacteria and Eschrichia coli cells. The invention also relates to bacterial hosts containing such DNA vectors, and further relates to vaccines containing such bacteria.

Mycobacterium bovis BCG (hereinafter BCG) has been used for many years as a vaccine against tuberculosis (TB). The vaccination programme has been extremely effective in controlling human TB firstly because BCG stimulates long term cell-mediated immunity and secondly because it has had an outstanding safety record. These characteristics make BCG a good candidate to form the basis of a live delivery system for recombinant vaccines.

A number of problems are associated with DNA manipulations involving mycobacteria. These include very few (one) plasmid vectors, poor growth rates of mycobacteria and low transformation rates when compared to bacterium such as E. coli. Given these problems, it is desirable to produce a shuttle vector which is capable of replication in a standard bacterial work horse such as E. coli for day to day genetic manipulations, and is

further capable of replication in Mycobacterium at or near the final stage of genetic manipulation. In this way, genes can be inserted into mycobacteria and time delays associated with Mycobacterium growth and transformation can be largely avoided. Gicquel-Sanzey et al. (Acta Lepologica 1989, 7, Suppl (1): 208-211) have described a mycobacteria-E. coli plasmid shuttle vector known as pAL8 which comprises two origins of replication, the first for mycobacteria and the second for E. coli. Multiple origins of replication have been necessary due to the evolutionary distance between mycobacteria and E. coli such that a mycobacterial plasmid having a mycobacterial origin is not capable of growth in E. coli and vice versa. Such vectors suffer from the problem that they are a considerable size due to the inclusion of two origins of replication, this decreasing cloning efficiency, the size of desired DNA fragments which may be inserted into such plasmids, and also increasing the number of unique restriction sites which may be introduced into the plasmids.

The present applicant has overcome problems associated with prior art shuttle vectors by providing a DNA shuttle vector which carries a single origin of replication which confers the ability of the vector to replicate in mycobacteria and E. coli cells. In a particularly preferred aspect of this invention as will be described hereinafter, the replication region corresponds to that of the corynebacterial plasmid pNG2 or fragments thereof.

In accordance with a first aspect of this invention, there is provided a DNA shuttle vector carrying a replication region comprising a single origin of replication, said origin of replication allowing replication of said vector in Mycobacteria and E. coli cells; and a selectable marker. The shuttle vector may additionally comprise a nucleotide sequence containing one or more restriction sites for the insertion of a

desired nucleotide sequence.

Preferably, the replication region corresponds to the replication region of the corynebacteria plasmid pNG2 or a fragment thereof which permits replication of said vector in mycobacteria and E. coli.

Surprisingly, the replication region of plasmid pNG2 confers the ability of replication in various bacterial species, apart from Corynebacteria.

The DNA shuttle vector may further comprise a promoter with one or more restriction endonuclease sites downstream of said promoter, such that when a nucleotide sequence is inserted into one or more of these sites, the promoter allows DNA transcription to proceed. The DNA shuttle vector may contain multiple promoters and downstream restriction endonuclease sites.

The term "shuttle vector" as used herein includes plasmid DNA which may be double-stranded linear or double-stranded circular. The shuttle vector may be introduced into a bacterial cell by any number of techniques well known in the art, such as conjugation, mobilisation, transformation, transfection, transduction or electroporation.

The term "selectable marker" as used herein refers to any selectable characteristic provided by or encoded for, by a nucleotide sequence. Suitable detectable markers include resistance to antibiotics or enzymes or immunologically detectable proteins or chemicals capable of causing a detectable reaction when provided with a suitable substrate. Examples include resistance to ampicillin, streptomycin, penicillin, hygromycin, kanamycin, and the like, β-galactosidase, urease, alkaline phosphatase and the like.

The term "promoter" is used in its broadest sense and refers to any nucleotide sequence which binds to RNA polymerase and which directs the transcription of nucleotide sequences downstream (3' "or operably linked") to the promoter. Suitable promoters include prokaryotic

promoters such as the P1 promoter of bacteriophage lambda, trp promoter, lac promoter, kanamycin resistance promoters of transposon Tn903 and transposon Tn5, mycobacterial promoters such as that of the promoter of the common mycobacterial 65Kd antigen, ribosomal RNA promoter of mycobacteria, promoters of M.bovis antigens MPB70, MPB59 and MPB64 and the like, hybrids between eukaryotic and prokaryotic promoters, and eukaryotic promoters such as the metallothionine promoter, growth hormone promoter, and the like.

Restriction endonuclease sites provided on the vector may correspond to the cleavage of one or more known restriction endonucleases, such as EcoR1, BamH1, Pst1, Cla1, Kpn1, HindIII, HincI and HincII, and the like. Restriction endonuclease sites may be provided in the form of one or more polylinkers which contain a number of closely grouped restriction endonuclease sites.

Nucleotide sequences of interest may be inserted into the endonuclease cleavage sites provided on the vector by ligation of DNA fragments having complementary "sticky ends" to allow annealing thereof, or by ligation of nucleotide sequence having "flush" ends (ends having no unpaired nucleotides) by methods well known in the art, and described for example, in Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 2nd Edition, 1989). A nucleotide sequence for insertion into the vector of this invention may include a promoter to direct transcription of downstream (3') sequences. Promoters may be the natural promoter of the gene to be transcribed or may be a different promoter. As previously mentioned, the shuttle vector of this invention may itself contain one or more promoters upstream (5') from nucleotide sequences encoding one or more restriction endonuclease cleavage sites. In such an embodiment, a desired nucleotide sequence lacking a promoter may be inserted into the vector with transcription of the desired nucleotide

sequence being driven by the promoter resident on the vector.

Nucleotide sequences for insertion into the vector of this invention may, for example, encode one or more antigens from disease causing bacteria, viruses or parasites, such as Taenia ovis, rotavirus, Babesia bovis, Mycobacterium bovis, Mycobacterium tuberculosis, Mycobacterium leprae, Mybacterium paratuberculosis, Bacteriodes nodosus or Bordetella. Desired nucleotide sequences may also encode hormones such as LHRH, growth hormone or epitopes or analogues thereof; or DNA sequences capable of recombination with nucleotide sequences within a bacterial host cell; or nucleotide sequences capable of mutagenising DNA sequences within a host cell. Where the shuttle vector of this invention functions as an expression vector, nucleotide sequences encoding desired products may include a signal or leader sequence to allow insertion into membranes of a suitable host cell or secretion from a host cell. Absence of a secretory leader will cause the accumulation of antigen or other protein product within the cytoplasm of a bacterial host cell, where it may be recovered by well known methods.

In accordance with a specific embodiment of this invention there is provided a DNA shuttle vector pEP2, said vector having a size of about 3.1 kb, as determined by agarose gel electrophoresis, a replication region of about 1.85 kb comprising a single origin of replication derived from the Corynebacterium replicon pNG2, an antibiotic resistance gene to kanamycin, and a nucleotide sequence containing a number of restriction endonuclease cleavage sites for the insertion of a DNA sequence of interest. The 4.5 kbr plasmid pEP3 contains the same sequences of replication as pEP2 plus a marker encoding hygromycin resistance effective in both E.coli and mycobacteria.

Shuttle vectors of this invention are capable of

replication in species of both gram negative and gram positive hosts, such as Mycobacterium, E. coli, Corynebacterium, and Actinomycetes, as defined in Bergey's Manual of Determinative Bacteriology, 8th Edition, pp. 599-861. The vectors of this invention do not appear to replicate in Bacillus species. Any bacterial strain may be readily tested according to methods well known in the art, to ascertain whether or not the shuttle vector of this invention is capable of replication therein. Advantageously, the shuttle vectors of this invention are capable of replication to high copy numbers in bacterial strains such as in the attenuated strain of Mycobacterium bovis BCG, which as previously stated has been extensively used in vaccination programmes throughout the world, and is a potent adjuvant, which stimulates long-term cell mediated immunity.

Shuttle vectors incorporating the origin of replication of pNG2 are particularly efficient in organisms of the genus Corynebacterium.

In accordance with a further aspect of this invention, there is provided a bacterial host which contains a shuttle vector as herein defined. The shuttle vector may be present as a single copy, or more preferably as multiple copies thereof within the bacterial host. Preferably, but in no way limiting the invention, the bacterial host is a Mycobacteria, Corynebacteria or E. coli strain, such as C. pseudotuberculosis, M. smegmatis, and M. bovis BCG. The vector may be used to deliver antigen or other protein genes into the bacterial host for expression thereof, as an excreted product from the host cell as previously described. Proteins may be expressed while residing on the plasmid vector or after recombination or insertion into the chromosome. Alternatively, expressed products may be inserted into or associated with the host cell membrane or cell wall or resident within the host cell

itself. Bacterial hosts expressing desired antigens may be provided as vaccines, for example, as M. bovis BCG, expressing a desired antigen. On immunisation, an immune response would be mounted to the host, such as M. bovis BCG as well as the desired antigen of the disease causing bacterium, virus or parasite.

In a further aspect of this invention there is provided a polypeptide when expressed by a bacterial host cell containing a shuttle vector as herein defined.

This invention contemplates deletions or insertions of nucleotide sequences to or from the replication region of pNG2 as long as such modifications do not prevent the ability of such sequences to confer replication in Mycobacterium and E. coli. Techniques for insertion or deletion of nucleotide sequences are well known in the art. Mutants could be readily tested for the ability to confer replication in gram negative and gram positive host cells, such as E. coli and corynebacteria.

This invention also extends to replication region of pNG2 itself or fragments thereof, which, on insertion into suitable vector are capable of permitting replication of said vector mycobacteria and E. coli.

A culture of Eschrichia coli containing plasmid pEP2 was deposited under the terms and conditions of the Budapest Treaty at the Australian Government Analytical Laboratory (AGAL), Pymble, New South Wales, Australia on 23rd February, 1990 and accorded Accession No. N90/007080.

This invention will now be illustrated with reference to the following non-limiting Figures and Examples.

## FIGURES

**Figure 1** shows the construction of plasmid pEP2. Plasmid pNG2 (14.5) was digested with EcoRI and the largest fragment ligated to the Kan$^r$ cartridge of pUC4K. Following electroporation into E.coli the resulting plasmid DNA was extracted and partially digested with

PstI, and then religated and again electroporated with E.coli. One of the resulting Kan$^r$ colonies contained plasmid pEP2. Restriction siles: B(BamH1), E(EcoRI), H(HindIII), Hc(HincII), P(PstI), S(SalI). Kan refers to kanamycin resistance gene from pUC4K.

**Figure 2A** is a circular map of the Mycobacterium-E. coli shuttle vector pEP2. The restriction map is given only for relevant cleavage sites: B (BamHI), E. (EcoRI), H (HindIII), Hc (HincII), K (KpnI), p (PstI), S (SalI). Kanamycin resistance gene \\\\\ from pUC4K.

**B** Sequencing strategy. Arrows indicate the extent and direction of sequence generated from restriction fragments cloned in M13 vectors. X denotes sequence derived using oligonucleotide primers.

**Figure 3** is a complete nucleotide sequence of the replication region of pEP2. Relevant restriction sites are marked for comparison with Figure 1 (E (EcoRI), H (HindIII), Hc (HincII), K (KpnI). IR and DR, inverted and direct repeat sequences respectively; RBS, putative ribosome binding site; ------> <------ dyad symmetry associated with putative rho-dependent transcriptional terminator, T. ORFA, major open reading frame.

**Figure 4** shows an agrose gel (right hand plate) and a Southern blot (left hand plate) of that gel probed with plasmid pEP2 extracted from E.coli. Tracks contain (A) Undigested pEP2 DNA from E.coli 500ng, (B) Pst 1 digested pEP2 DNA from E.coli 200ng, (C) PstI digested whole DNA extract of M. bovis BCG pEP2, 2.5ug, (D) PstI digested M. bovis BCG DNA, 2.5 ug (E) Undigested DNA extract of M. bovis BCG pEP2, 2.5ug, (F) Undigested DNA extract of M. bovis BCG, 2.5ug, (G) HindIII digested lambda DNA markers.

## EXAMPLE 1

Recombinant DNA Procedures:

Unless otherwise specified herein, manipulation of recombinant molecules and the preparation of solutions are by standard known techniques. Such techniques are

described in Sambrook et al. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor [1989], pp. 1-500.

**Bacterial Strains and Plasmids:**

Mycobacterium bovis BCG variant CSL was obtained as lyophylised human vaccine from the Commonwealth Serum Laboratories (CSL) Australia. M. smegmatis and M. pheli were obtained from the Fairfield Hospital, Melbourne, Australia and Escherichia JM109 was obtained from Promega (Madison, Wisconnsin, U.S.A.). Plasmids pNG2 (Serwold-Davis et al. 1987, Proc. Natl. Acad. Sci. USA 84: 4964-4968) and pPB3 was obtained from Dr. Philip Bird (Monash University Faculty of Medicine, Alfred Hospital, Melbourne). Plasmid pAL8 (Gicquel-Sanzey et al. 1989 Acta Leprol. 7: 207-211) was obtained from Dr. Brigette Gicquel-Sanzey (Pasteur Institute, Paris) and plasmid pUGC4K was purchased from Pharmacia LKB (Uppsala, Sweden).

**Media:**

E. coli strains were grown Luria broth, (LB:10 grams tryptone, 5 grams yeast extract, 10 grams NaCl per litre). Coryneform bacteria were cultured in LB media (Oxoyd) and mycobacterium species were grown in Dubos or 7H11 media (Oxoid, Australia).

**Electroporation of Bacteria:**

Mycobacterium species and Corynebacterium pseudo-tuberculosis were electroporated according to Lugosi et al. (Tubercule 70: 159-170 [1989]) using a Gene Pulser commercially available from Bio-Rad Laboratories Inc. Transformants were selected on 7H11 or nutrient media containing 100 µg kanamycin per ml or 200µg hygromyan B per ml.

**DNA Isolation and Hybridization Analysis:**

DNA was extracted from Mycobacterium using a modification of a method used to isolate DNA from yeast (Mann and Jeffrey, Anal. Biochem. 1981, 178: 82-87). Mycobacterial cells were harvested from 400 mls of Dubos

broth by centrifugation, resuspended in 5 ml of TE buffer and heat-killed by treatment at 70°C for 1 hour. Glass beads (3-6 mm) were added and the mix vortexed vigorously for a minimum of 30 seconds to disperse the bacteria. Bacterial suspensions were then transferred to liquid nitrogen in a mortar pre-cooled in a bath of liquid nitrogen and crushed into a fine powder using a pre-cooled pestle. The crushing of cells was performed in a biohazard hood. Frozen crushed cells were added in small portions (spatula loads) to 15 ml of lysis buffer (6.6 mM Tris, 30 mM EDTA, 1.2% w/v sodium lauroylsarkosinate). Five mg of protease K was added and the mixture incubated for 90 minutes at 37°C. The mix was then extracted with an equal volume of phenol-chloroform and the aqueous phase precipitated with isopropanol at 4°C for 10 minutes. The pellet was dissolved in 1.0 ml of TE buffer and extracted with phenol-chloroform and then water-saturated ether prior to ethanol precipitation and resuspension in TE.

Genomic DNA was isolated from C. pseudotuberculosis as previously described (Hodgson et al., 1990).

pNG2RI DNA was digested with various restriction enzymes and the fragments Southern blotted (Reid et al.) to Hybond N (Amersham) nylon filters. Filters were hybridised overnight at 37°C with pEP2 labelled with 32P using random hexamer primers, washed at increasing stringency as necessary (up to 65°C) and exposed to X-ray film (Fuji RX). Restriction digested total genomic DNA isolated from M. bovis BCG and that transformed with pEP2 was Southern blotted to nylon and probed with labelled pEP2 as described above. Other transformed Mycobacterium species were analysed with the pEP2 probe using DNA dot blot hybridisation. C. pseudotuberculosis transformants were analysed in the same fashion.

Total cell DNA was used to isolate and transform E.coli to kanamycin or hygromycin resistance.

DNA Sequence Analysis:

Nucleotide sequence analysis was determined by the dideoxy chain termination method (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA 74: 5463-5467) using modified T7 DNA polymerase (Pharmacia LKB, Uppsula, Sweden). The complete nucleotide sequence of pEP2 was derived on both strands using universal or oligonucleotide primers (Figure 1B). The latter was synthesized using a Gene Assembler plus DNA Synthesizer (Pharmacia LKB, Supra). DNA and amino acid sequence data were collated and an analysed using the DNASIS and PROSIS software packages (Pharmacia LKB).

EXAMPLE 2

Shuttle Vector Construction and Analysis Thereof:

The 1.3 kb EcoR1 fragment from pUC4K carrying the kanamycin resistance gene was ligated to pNG2 DNA digested with EcoR1. The ligation mix was electroporated into E.coli JM109 and transformant selected on LB plates supplemented with 50µg kanamycin per ml. All transformants contained plasmids with the 9.5 kb pNG2 EcoR1 fragment. Plasmid DNA (2 µg) from one of these clones (pNG2RI) was partially digested with PstI and blunted using T4DNA polymerase. DNA fragments smaller than 10 kb were purified from a 1% agarose gel and used to transform E. coli JM109 to kanamycin resistance. A restriction map of a resulting plasmid (pEP2) isolated from a transformant was derived using standard procedures (Sambrook et al., Supra) and is shown in Figure 1A.

Plasmid pEP2 has a molecular weight of 3.1 kb as determined by agarose gel electrophoresis. This plasmid retains one of the pUC4K polylinkers and hence has unique PstI, SalI, BamH1 and EcoRI sites. Hybridization analysis shows that plasmids pEP2 and pNG2RI (pNG2 after digestion with EcoRI) both contain an 800 bp HindIII fragment. Southern blotting of digests of sub-clones of pNG2 with the pEP2 plasmid showed that the region of PNG2 marked in Figure 1 was that incorporated in the pEP2

plasmid, and thus the area containing the origin of replication.

To determine whether hygromycin resistance could be used as a selectable marker in Mycobacteria species and C. pseudotuberculosis a hygromycin phosphotransferase gene (hph) was cloned into the Mycobacterium shuttle vector pEP.

To clone the hygromycin phosphotransferase gene (hph) from pPB3 into pEP2, pUC4K DNA was linearised with XhoI and then blunted using the Klenow fragment of DNA polymerase I. pPB3 DNA was digested for 30s on ice using 5 units each of AluI, HaeIII and RsaI. Fragments 2.0kb in size were gel purified (Geneclean, Bresatech) and ligated overnight with the linear, blunted pUC4K DNA. The ligation mix was electroporated into JM109 and recombinants were selected on LB plates supplemented with 150ug hygromycin B (Sigma) per ml. A 1.7kb SalI-ClaI insert from a pUC4K chimera was ligated into the SalI-ClaI site within the kanamycin resistance gene of pEP2. JM109 was transformed to hygromycin resistance with the ligation mix as described above and a restriction map of a hygromycin resistance plasmid (pEP3) was derived. The plasmid pEP3 has a unique SalI site and is approximately 4.5 kb in size (Fig. 2).

The nucleotide sequence of pEP2 excluding the kanamycin resistant gene is presented in Figure 3. Examination of the DNA sequence revealed a single open reading frame (ORF). A number of potential translational start codons can be identified within this region but only one is preceded by a putative ribosome binding site (Figure 2). Although the sequence upstream of this ORF (ORFA) does not possess an E. coli consensus promoter, a putative rho-dependent transcriptional terminator was found downstream of the stop codon (Figure 2). This putative terminator has a 90% match to the TAATCAATAT consensus sequence (Ryder et al., Initiation of DNA Replication [1981], Academic Press, New York) and as has

been described for other rho-dependent terminators (Rosenberg et al., Nature 272, 414-428 [1978]), and is preceded by a region of dyad symmetry (Figure 2). ORFA is therefore capable of coding for a 28kDa protein, a size consistent with that reported for other Rep proteins.

In addition to the predicted size of the ORFA protein, further evidence to suggest that we have identified the legitimate translated region arises from an examination of the derived amino acid sequence. Firstly, the codon bias for the putative ORFA product (Phe, TTC; Asp, GAC; Arg, CGC; Ile, ATC; Val, GTC; Ala, Gcc; Thr, ACC) is the same as that for other Corynebacterium proteins. Secondly, the predicted protein encoded by ORFA is highly basic in nature (19% basic residues) which is a characteristic of Rep proteins (and other DNA binding proteins) thought to be important in the role they play in replication and incompatibility.

Database searches were performed using both the complete 1.85kb nucleotide sequence and the predicted amino acid sequence of ORFA, however no significant homologies were found. In addition, more detailed analyses revealed no similarities between the pEP2 Rep region and either of the potentially related plasmids from Corynebacterium (pBL1, Martin et al., Biotechnol. 5: 137-146 [1987]) and Mycobacterium (pAL5000, Rauzier et al., Gene II: 315-321 [1988]).

Plasmid replication regions invariably have an origin of replication. Most commonly, origins are located in non-coding regions, possess clusters of direct and inverted repeats and may be preceded by A+T rich sequences (Kamio et al., J. Bacteriol. 258: 307-312 [1984], Scott et al., Microbiol. Rev. 48: 1-23 [1984], Rosen et al., Mol. Gen. Genet. 179: 527-537 [1980], Rauzier et al., Gene 71: 315-321 [1988]). The region upstream of ORFA contains no ORFs and possesses a number of direct and inverted repeat DNA sequences (Figure 2).

In addition, the region between nt 86 and 171 is 63.5% A+T compared with an average of 45% for the entire Rep region (Figure 2). We therefore believe this to be the pEP2 origin of replication.

The replication region of pEP2 is capable of encoding a single 28kDa protein and possesses a single origin allowing plasmid replication in both Gram-positive and negative bacteria. pEP2 is therefore a unique shuttle vector and should be a useful tool, for example, in the genetic analysis of Mycobacterium and in the development of M. bovis BCG as a live recombinant vaccine.

Electroporation of Mycobacteria with pEP2 and pEP3:

Electroporation of Mycobacterium species with pEP2 DNA resulted in transformation to kanamycin resistance respectively (Table 1). To confirm that the plasmids were present in the mycobacteria, total cellular DNA preparations were made from the kanamycin resistant transformants. Figure 4 shows the results of agarose gel and Southern blot analysis of total cell DNA extracted from BCG CSL strains that had been transformed with pEP2. The pEP2 plasmid is clearly present in the transformed strains. Furthermore, the relative intensity of the gel bands suggests that the pEP plasmids replicate to high copy number in these bacteria. In addition, when total genomic DNA isolated from the drug resistant transformants was used to electroporate E. coli, approximately 1.0 X $10^5$ kanamycin resistant clones were obtained per ug DNA. Taken together these data suggest that the pEP2 replicon promotes stable plasmid replication to high copy number in these bacteria.

Plasmid pEP3 was capable of transforming M. smegmatis and M. bovis BCG CSL to resistance to at least 200ug hygromycin per ml (Table 1). Total cellular DNA isolated from pEP3 transformed M. smegmatis was capable of transforming E. coli to hygromycin resistance. This shows that the php gene encoding hygromycin resistance is

effective in both mycobacteria and E. coli and thereby constitutes a useful marker for these bacteria.

**TABLE 1**

**ELECTROPORATION OF MYCOBACTERIA AND RELATED SPECIES**

| Bacterial Species | Plasmid electroporation efficiency: $Kan^r$ CFU per microgram of plasmid DNA | | |
|---|---|---|---|
| | pEP2 | pAL8 | pEP3 |
| Mycobacterium phlei | --- | $10^3$<br>$10^2$ | $10^1$ |
| Mycobacterium smegmatis | $10^2$<br>$10^1$ | $10^1$ | $10^2$ |
| Mycobacterium bovis | $10^2$<br>$10^2$ | $10^2$<br>$10^3$ | N/D |
| BCG var CSL | $10^2$ | $10^4$ | $10^1$ |
| Corynebacterium ovis ( pseudotuberculosis ) | $10^3$<br>$10^4$ | --- | $10^2$ |

N/D = Not done, ---= no transformation to resistant phenotype
Different figures for each electroporation represent the result of separate experiments.

**Electroporation of Other Bacterial Species with pEP2 and pEP3:**

To determine the host range of the pEP replicon, pEP2 and 3 plasmid DNA was electroporated into Cornybacterium pseudotuberculosis. Transformation occurred in C. pseudo tuberculosis (Table 1). These results indicate that the pEP2 plasmids have a wide host range.

**EXAMPLE 3**

**Generation and Expression of pEP2 Constructs:**

A PCR derived DNA fragment carrying the promoter from the Mycobacterial 65kDa heat shock protein (0.6Kb) was cloned into the Pstl-BamHl sites of pEP2, generating a plasmid referred to as pEP5. This promoter is known to function in E. coli as well as Mycobacteria and is therefore useful in this shuttle vector expression system.

A gene encoding for chloramphenicol-acetyl-transferase (CAT) was cloned into the BamHl site of pEP5. Expression of the CAT gene driven by the 65kDa promoter was detected using the Pharmacia (Registered trademark, Pharmacia, Pitcataway, N.J., U.S.A.) CAT detection kit. According to this assay the 64kDa promoter has a strength comparable with the induced lac promoter in both E. coli and C. pseudotuberculosis.

MPB70 is the major secreted protein of M. bovis and a component of PPD. This gene and its signal sequence was incorporated into the pEP2 expression system. A truncated form of the MPB70 gene was generated by PCR removing its promoter but leaving its ribosome binding site (RBS) intact. This was cloned into pEP2 as a Pstl-BamHl fragment (0.5Kb). The 65kDa promoter was then cloned upstream of the MPB70 BS on a Pstl-Scal fragment.

After electroporation into E. coli and C. pseudotuberculosis, expression was tested for by western blots and Elisas. This construct did not express in E.

coli. It did however express in C. pseudotuberculosis with product detected in both the solicate and culture filtrate. Ths indicated that the MPB70 RBS was inactive in E. coli yet was recognised and functional in C. pseudotuberculosis.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within its spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

CLAIMS:

1. A DNA shuttle vector carrying a replication region comprising a single origin of replication, said origin of replication allowing replication of said vector in Mycobateria and E.coli cells; and a selectable marker.

2. A shuttle vector according to claim 1 comprising a first nucleotide sequence operably linked to said origin of replication, and containing one or more restriction endonuclease cleavage sites for the insertion of a desired second nucleotide sequence.

3. A shuttle vector according to any one of claims 1 or 2 which comprises one or more promoters having operably linked thereto a nucleic acid sequence encoding a desired polypeptide.

4. A shuttle vector according to claim 1 wherein said selectable marker confers resistance to an antibiotic or enzyme, or encodes an immunologically detectable protein or chemical compound capable of causing a detectable reaction when provided with a suitable substrate.

5. A shuttle vector according to claim 4, wherein said selectable marker confers resistance to ampicillin, streptomycin, penicillin, hygromycin or kanamycin.

6. A shuttle vector according to claim 4, wherein said selectable marker is a gene encoding β-galactosidase, urease or alkaline phosphotase.

7. A shuttle vector according to claim 3, wherein said promoter is selected from the P1 promoter of bacteriophage lambda, trp promoter, lac promoter,

kanamycin resistance promoters of transposon Tn903 or transposon Tn5, mycobacteria promoter of the common mycobacterial 65Kd antigen, ribosomal RNA promoter of mycobacteria, promoter of M.bovis antigens MPB70, MPB59 and MPB64, metallothionine promoter, growth hormone promoter or hybrids between eukaryotic and prokaryotic promoters.

8. A shuttle vector according to claim 3, wherein said desired peptide encodes one or more antigens from disease causing bacteria, viruses or parasites.

9. A shuttle vector according to claim 8, wherein said one or more antigens are selected from antigens of Taenia ovis, rotavirus, Babesia bovis, Mycobacterium bovis, Mycobacterium tuberculosis, Mycobacterium leprae, Mybacterium paratuberculosis, Bacteriodes nodosus or Bordetella.

10. A shuttle vector according to claim 3, wherein said desired polypeptide is a hormone or an epitope thereof.

11. A shuttle vector according to claim 1, wherein said origin of replication comprises the replication region of the Corynebacterium plasmid pNG2 or a fragment thereof which permits replication of said vector in mycobacteria and E.coli.

12. A shuttle vector according to any one of claims 1 to 11, capable of replication in Actinomycetes.

13. A shuttle vector according to any one of claims 1 to 11, capable of replication in Corynebacteria.

14. A shuttle vector selected from pEP2 and pEP3.

15. A bacterial host cell containing a shuttle vector according to any one of claims 1 to 14.

16. A host cell according to claim 15 selected from Mycobacteria, E. coli, Corynebacteria, and Actinomycetes.

17. A host cell according to claim 15 selected from M. pheli, M. smegmatis, M. bovis BCG, E. coli JM109, C. pseudotuberculosis.

18. A DNA sequence encoding the origin of replication of Corynebacterium plasmid pNG2 or a fragment thereof which permits replication of a vector containing said DNA sequence in Mycobacteria and E. coli.

19. A DNA sequence according to claim 17 comprising an origin of replication operably linked to said origin of replication.

20. A DNA sequence according to claim 18 where the DNA sequence encodes a desired polypeptide is operably linked to said promoter.

21. A polypeptide expressed by a host cell wherein said polypeptide is encoded by a shuttle vector according to any one of claims 4 and 8 to 9, resident within said host cell.

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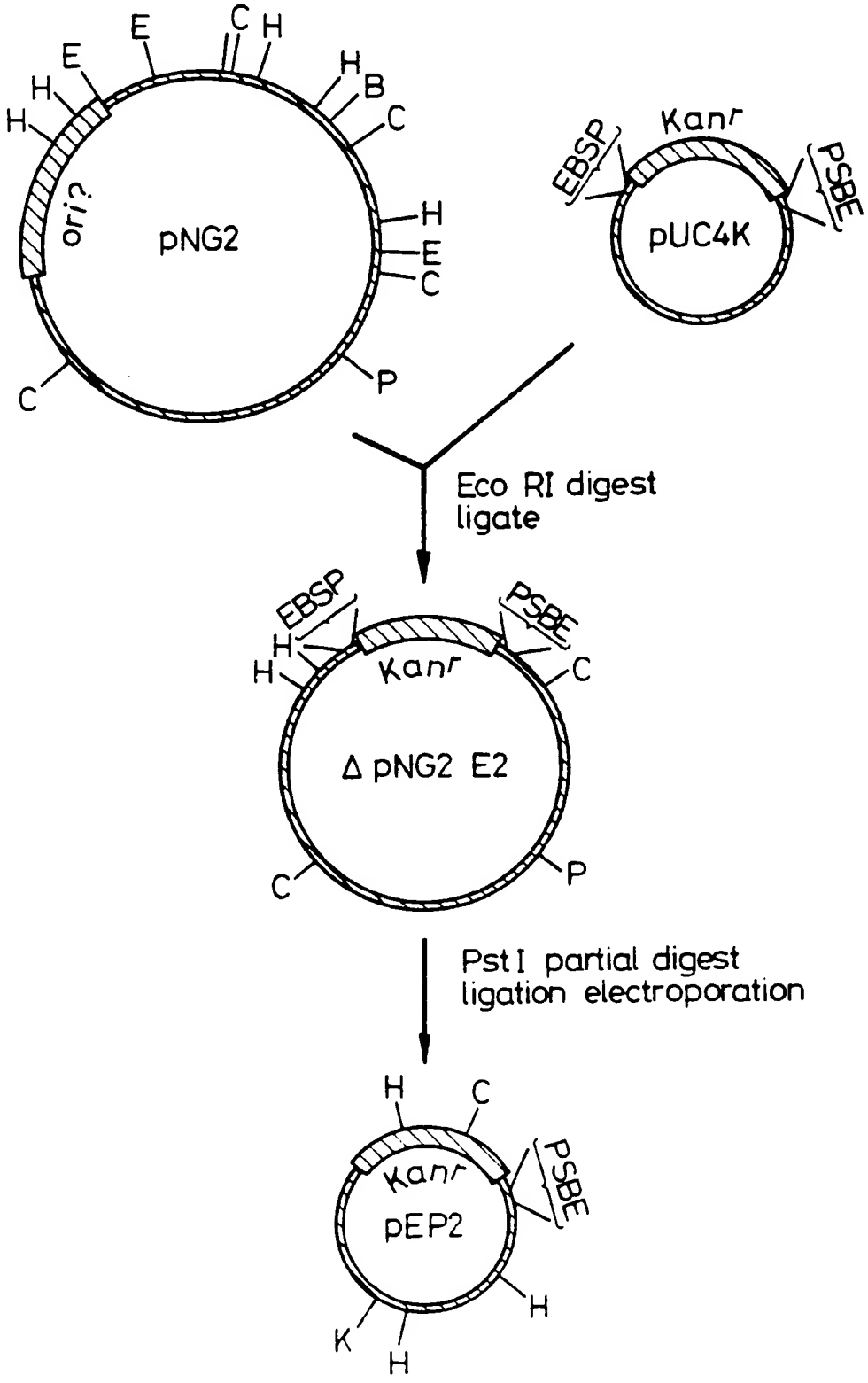
Fig. 1.


pNG2
ori?
pUC4K
Kanr
EBSP
PSBE
Eco RI digest
ligate
Δ pNG2 E2
Pst I partial digest
ligation electroporation
pEP2

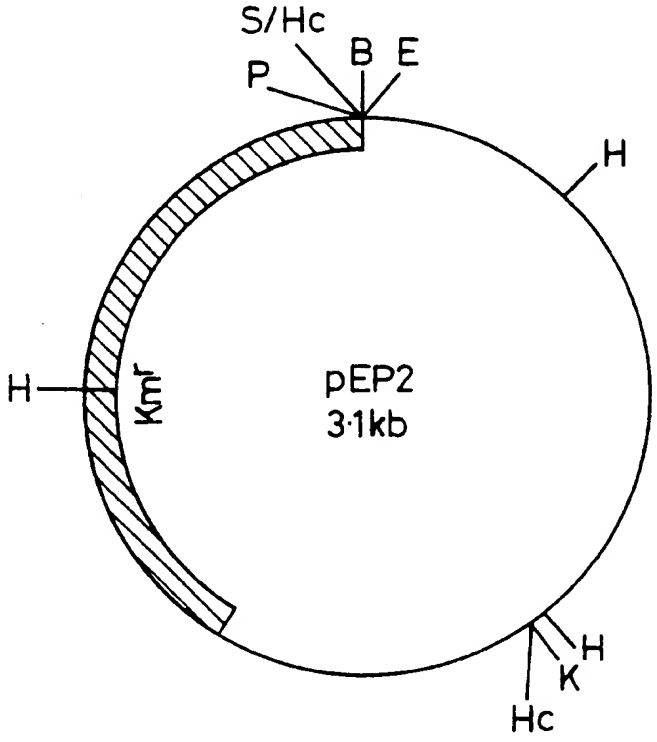



Fig.2A.

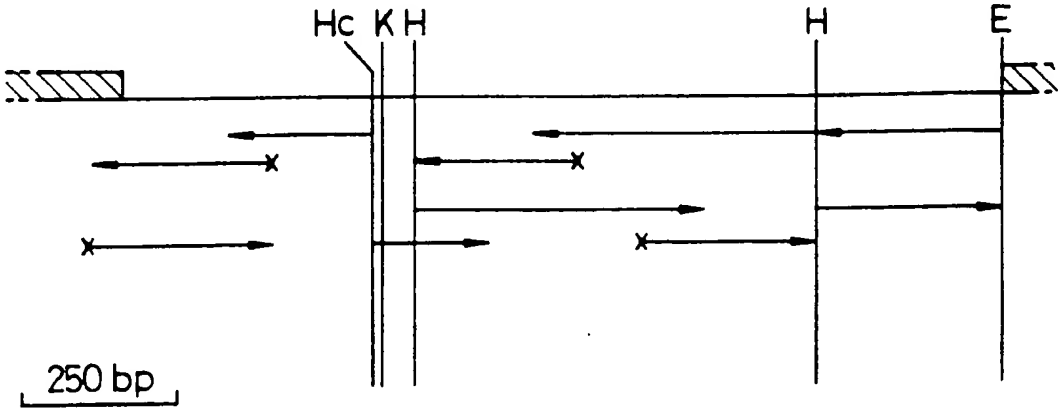
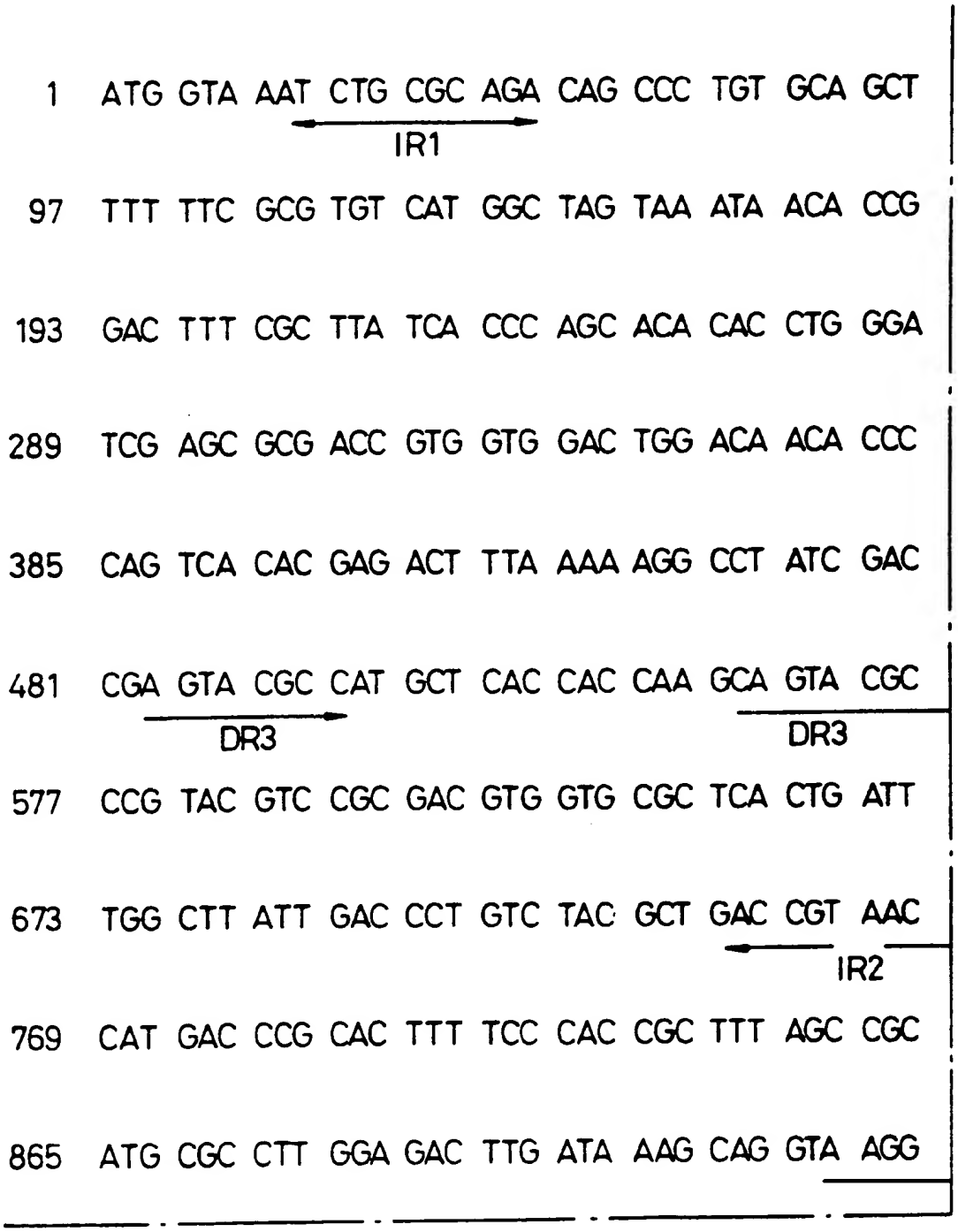



Fig.2B.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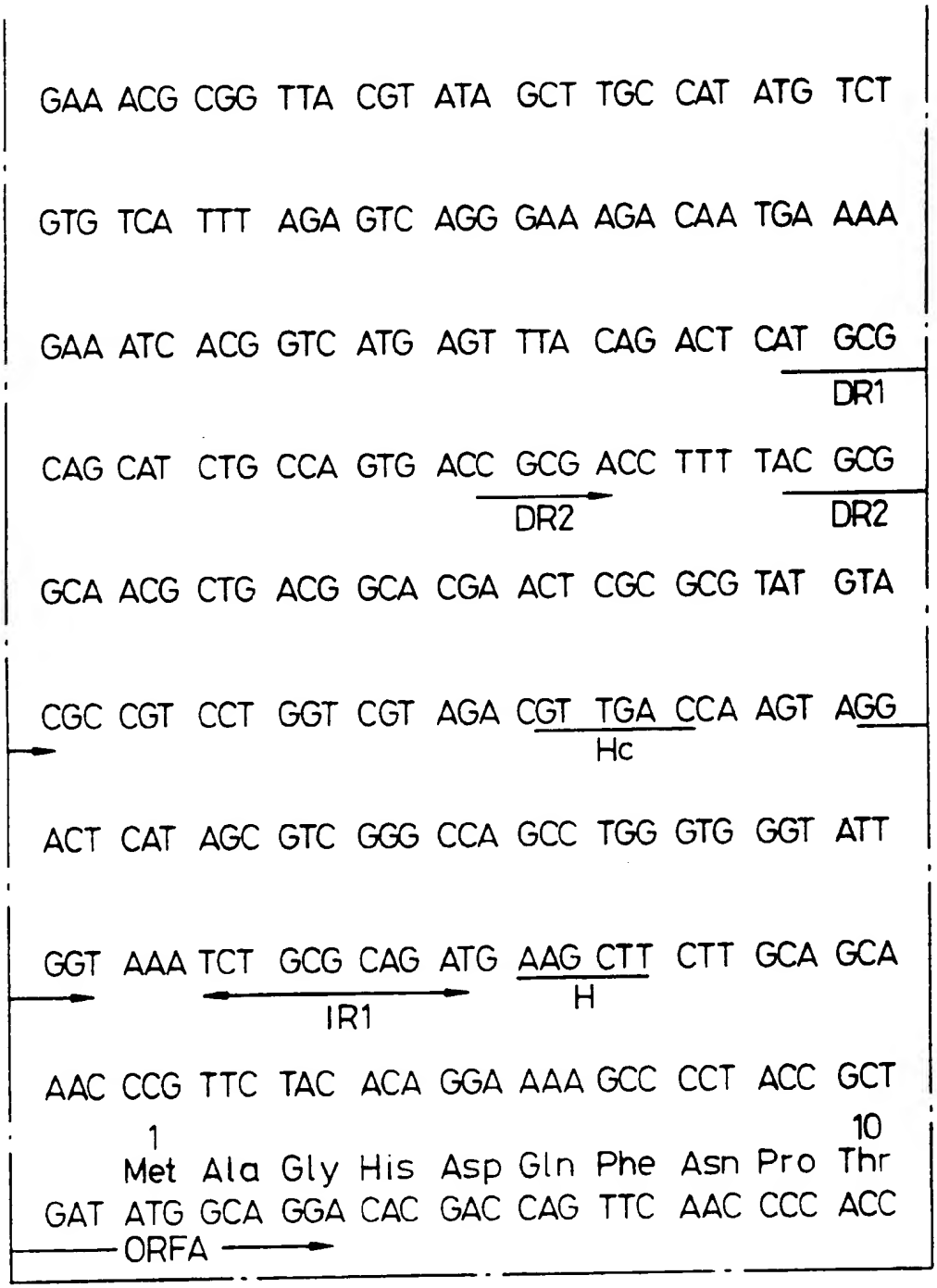
IR1
97 TTT TTC GCG TGT CAT GGC TAG TAA ATA ACA CCG
193 GAC TTT CGC TTA TCA CCC AGC ACA CAC CTG GGA
289 TCG AGC GCG ACC GTG GTG GAC TGG ACA ACA CCC
385 CAG TCA CAC GAG ACT TTA AAA AGG CCT ATC GAC
481 CGA GTA CGC CAT GCT CAC CAC CAA GCA GTA CGC
DR3
DR3
577 CCG TAC GTC CGC GAC GTG GTG CGC TCA CTG ATT
673 TGG CTT ATT GAC CCT GTC TAC GCT GAC CGT AAC
IR2
769 CAT GAC CCG CAC TTT TCC CAC CGC TTT AGC CGC
865 ATG CGC CTT GGA GAC TTG ATA AAG CAG GTA AGG

Fig. 3a.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GTG TCA TTT AGA GTC AGG GAA AGA CAA TGA AAA
GAA ATC ACG GTC ATG AGT TTA CAG ACT CAT GCG
DR1
CAG CAT CTG CCA GTG ACC GCG ACC TTT TAC GCG
DR2
DR2
GCA ACG CTG ACG GCA CGA ACT CGC GCG TAT GTA
CGC CGT CCT GGT CGT AGA CGT TGA CCA AGT AGG
Hc
ACT CAT AGC GTC GGG CCA GCC TGG GTG GGT ATT
GGT AAA TCT GCG CAG ATG AAG CTT CTT GCA GCA
IR1
H
AAC CCG TTC TAC ACA GGA AAA GCC CCT ACC GCT
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10
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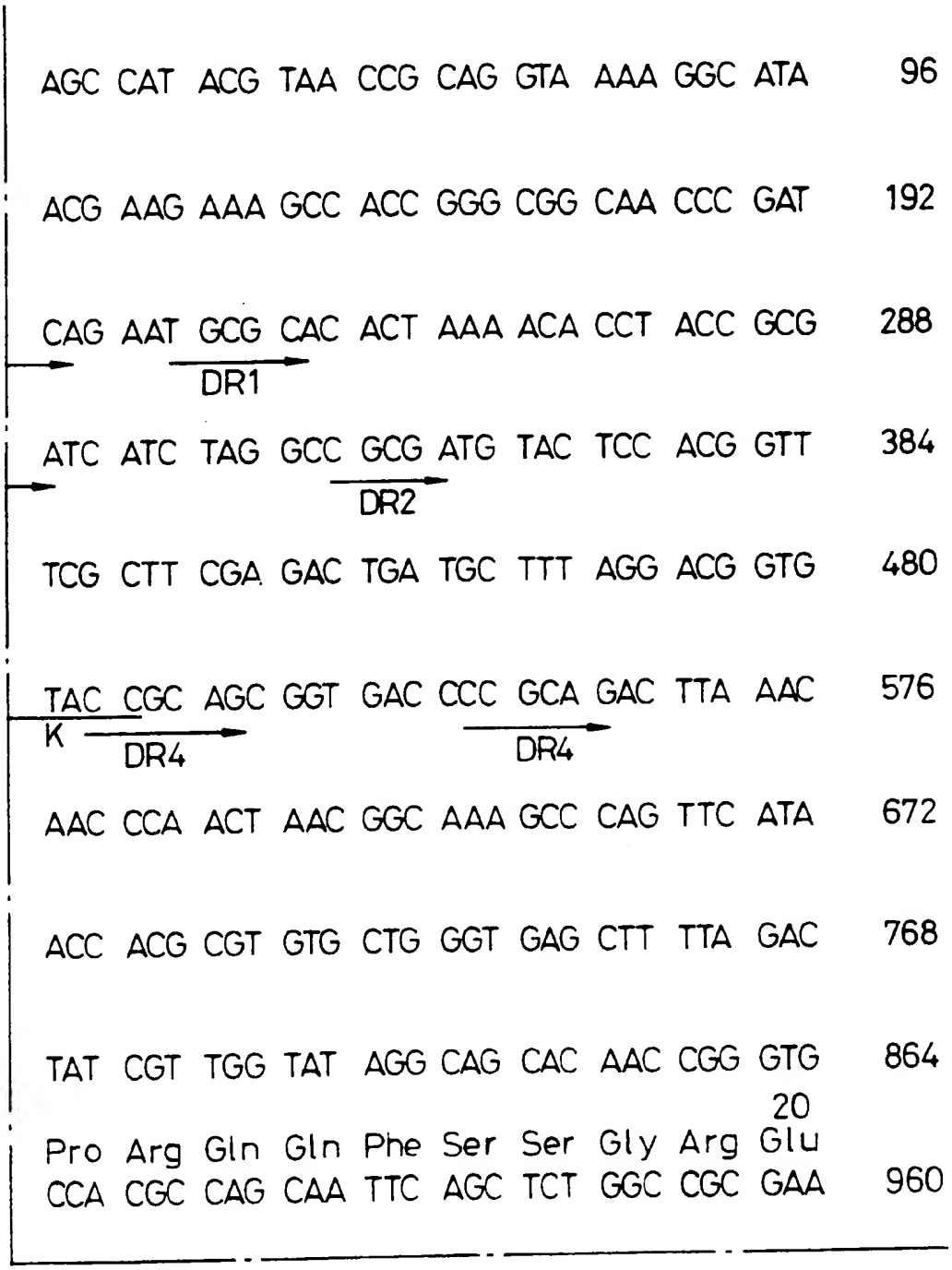
GAT ATG GCA GGA CAC GAC CAG TTC AAC CCC ACC
ORFA

Fig.3b.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ACG AAG AAA GCC ACC GGG CGG CAA CCC GAT 192
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DR1
ATC ATC TAG GCC GCG ATG TAC TCC ACG GTT 384
DR2
TCG CTT CGA GAC TGA TGC TTT AGG ACG GTG 480
TAC CGC AGC GGT GAC CCC GCA GAC TTA AAC 576
K
DR4
DR4
AAC CCA ACT AAC GGC AAA GCC CAG TTC ATA 672
ACC ACG CGT GTG CTG GGT GAG CTT TTA GAC 768
TAT CGT TGG TAT AGG CAG CAC AAC CGG GTG 864
20
Pro Arg Gln Gln Phe Ser Ser Gly Arg Glu
CCA CGC CAG CAA TTC AGC TCT GGC CGC GAA 960

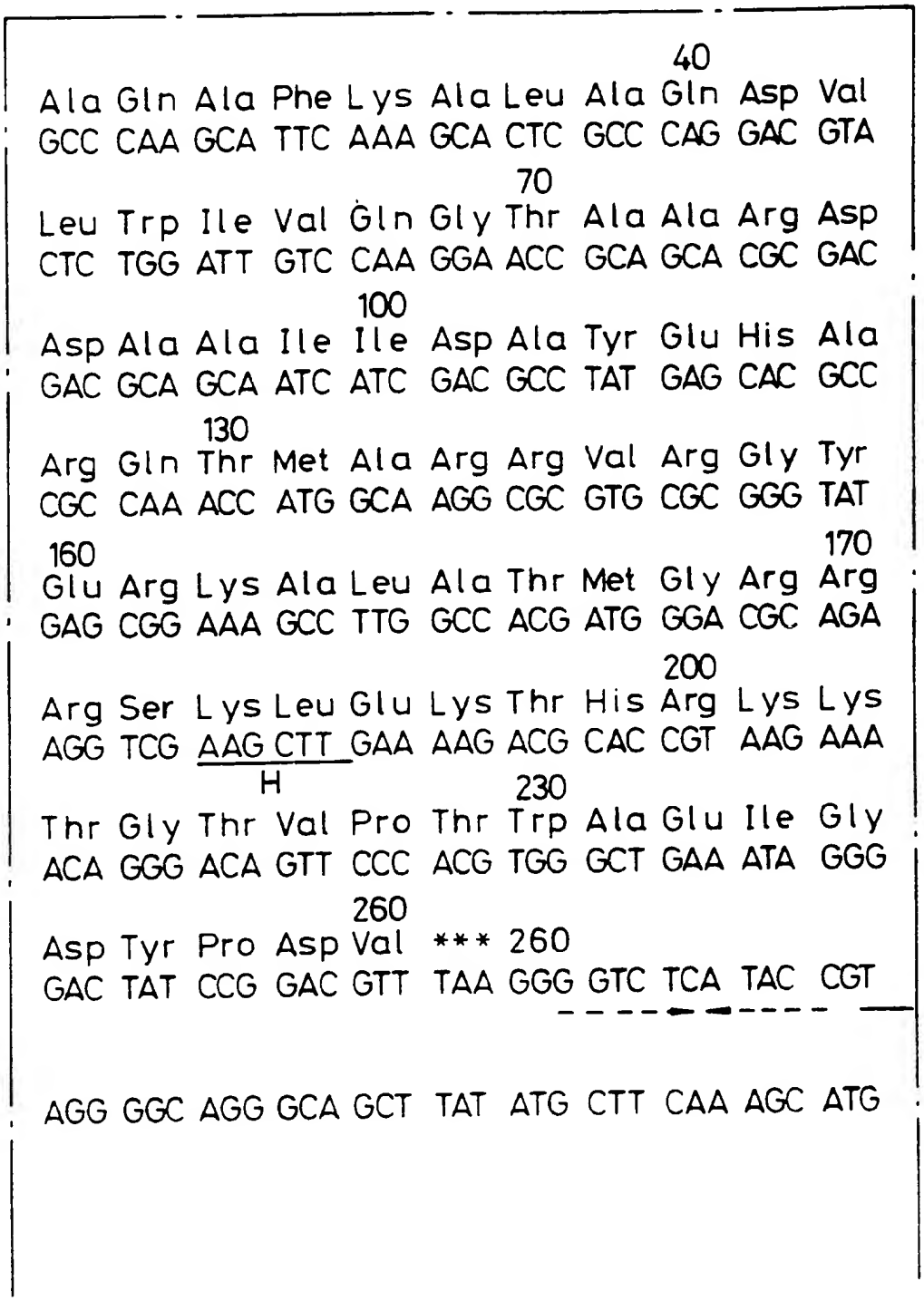
Fig. 3c.

```
                                                                  30
        Leu Ile Asn Ala Val Lys Thr Arg Arg Glu Glu
961     CTT ATC AAC GCG GTC AAG ACC CGC CGT GAA GAA
                                              60
        Tyr Asp Pro Glu Leu Ile Asp Gly Val Arg Val
1057    TAT GAC CCG GAA CTT ATC GAC GGT GTG CGT GTG
                                  90
        Ala Thr Val Ala Pro Ala Ser Gln Arg Leu Thr
1153    GCC ACC GTT GCG CCA GCA AGC CAA CGC CTG ACA
                      120
        Gly Arg Asp Asn Glu Met Pro Pro Met Arg Asp
1249    GGC CGC GAC AAC GAG ATG CCA CCC ATG CGC GAC
            150
        Gly Ser Asn Ala Pro Gly Lys Ala Thr Ser Ser
1345    GGC TCT AAC GCA CCA GGT AAA GCC ACC AGC AGC
                                                  190
        Thr Asp Pro Glu Gly Lys Tyr Ala Gln Ala Gln
1441    ACA GAC CCC GAG GGC AAA TAT GCG CAA GCA CAA
                                          220
        Ile Ser Gln Met Val Asn Asp Gln Tyr Phe Gln
1537    ATT AGC CAA ATG GTG AAC GAT CAG TAT TTC CAG
                                  250
        Ala Arg His Val Ala Glu Leu Lys Lys Ser Gly
1633    GCT AGG CAT GTC GCG GAG CTA AAG AAG AGC GGT


1729    AGT TAG ATA AAA CCT CAC TTG AAG AAA ACC TTG


1825    CTC CGC CAT AAC CTC ACC GAA TTC
                                E
```

Fig. 3d.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70
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CTC TGG ATT GTC CAA GGA ACC GCA GCA CGC GAC
100
Asp Ala Ala Ile Ile Asp Ala Tyr Glu His Ala
GAC GCA GCA ATC ATC GAC GCC TAT GAG CAC GCC
130
Arg Gln Thr Met Ala Arg Arg Val Arg Gly Tyr
CGC CAA ACC ATG GCA AGG CGC GTG CGC GGG TAT
160
170
Glu Arg Lys Ala Leu Ala Thr Met Gly Arg Arg
GAG CGG AAA GCC TTG GCC ACG ATG GGA CGC AGA
200
Arg Ser Lys Leu Glu Lys Thr His Arg Lys Lys
AGG TCG AAG CTT GAA AAG ACG CAC CGT AAG AAA
H
230
Thr Gly Thr Val Pro Thr Trp Ala Glu Ile Gly
ACA GGG ACA GTT CCC ACG TGG GCT GAA ATA GGG
260
Asp Tyr Pro Asp Val *** 260
GAC TAT CCG GAC GTT TAA GGG GTC TCA TAC CGT
AGG GGC AGG GCA GCT TAT ATG CTT CAA AGC ATG

Fig. 3e.

```
                                                  50
Asp Ala Glu Ile Ala Gly Gly Leu Asp Gln
GAC GCG GAA ATC GCC GGT GGT CTC GAC CAG    1056
                          80
Glu Gln Pro Leu Asp Met Arg Leu Arg Leu
GAA CAG CCT TTA GAC ATG CGC TTA AGA CTG    1152
               110
Tyr Asn Val Ala His Thr His Gly Gly Ala
TAC AAC GTC GCA CAC ACC CAC GGC GGT GCA    1248
     140
Val Ala Gln Ser Lys Ser Glu Thr Tyr Ser
GTC GCC CAA TCC AAG AGC GAG ACC TAC AGC    1344
                                   180
Gly Gly Gln Lys Ala Ala Gln Arg Trp Lys
GGC GGA CAA AAA GCC GCA CAA CGC TGG AAA    1440
                                  210
Lys Ala Gln Gly Arg Ser Thr Lys Ser Arg
AAG GCT CAA GGA CGA TCT ACG AAG TCC CGT    1536
                          240
Ala Glu Val Gly Val Ser Arg Ala Thr Val
GCA GAG GTA GGA GTC TCT CGC GCC ACG GTT    1632


AAG CAA TAT ACG GTT CCC CTG CCG TTA GGC    1728
     T

ACT TCC TCT GTT CTC CTA GAC CTC GCA ACC    1824
```

Fig. 3f.

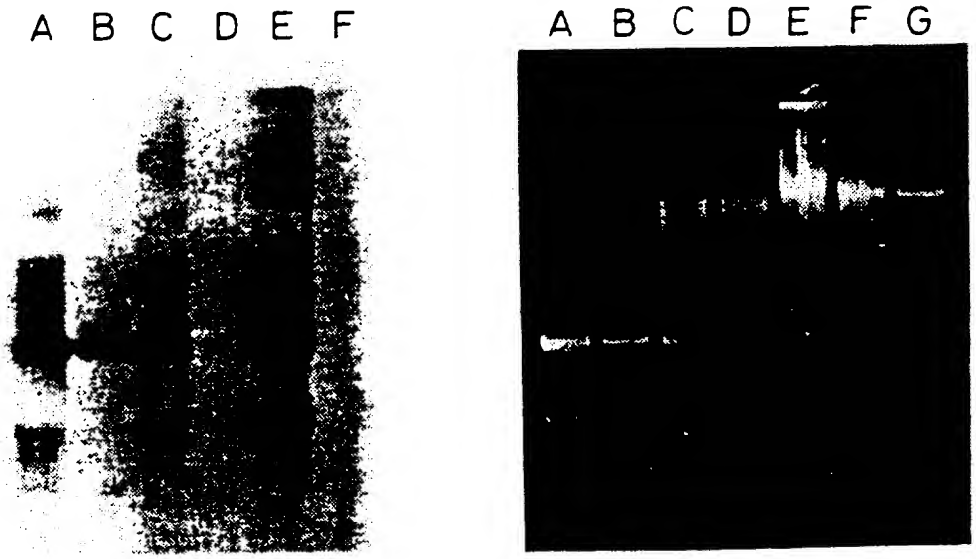


Fig.4.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 91/00064

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C12N 15/74, 15/76, 15/77

## II. FIELDS SEARCHED

Minimum Documentation Searched 7

| Classification System | Classification Symbols |
|---|---|
| WPI/WPIL )<br>Chem Abstr) | Keywords - MYCOBACTER:, VECTOR, PLASMID, SHUTTLE, CORYNEBACTER: |

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

BIOTECHNOLOGY DATABASE KEYWORDS (as above)
AU : C12N 15/74, 15/76, 15/77

## III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

| Category* | Citation of Document, [11] with indication, where appropriate, of the relevant passages 12 | Relevant to Claim No 13 |
|---|---|---|
| X | WO,A1, 90/00594 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 25 January 1990 (25.01.90) | (1-6,8-10,21) |
| X | WO,A, 88/06626 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 7 September 1988 (07.09.88) | (1-6,8-10,21) |
| A | US,A, 4952500 (UNIVERSITY OF GEORGIA RESEARCH FOUNDATION INC) 28 August 1990 (28.08.90) | (1-21) |
| A | WO,A1, 90/10701 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 20 September 1990 (20.09.90) | (1-21) |

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 7 June 1991 (07.06.91) | 7 June 1991 |
| International Searching Authority | Signature of Authorized Officer |
| Australian Patent Office | K. AYERS |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [ ] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[ ] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2.[ ] Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[ ] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1.[ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.[ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.[ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.